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FALSE PRECIPITIN BANDS IN OUCHTERLONY GEL DIFFUSION TESTS DUE TO FILTERABLE BACTERIAL FORMS

Werner A. Janssen
Michael J. Surgalla

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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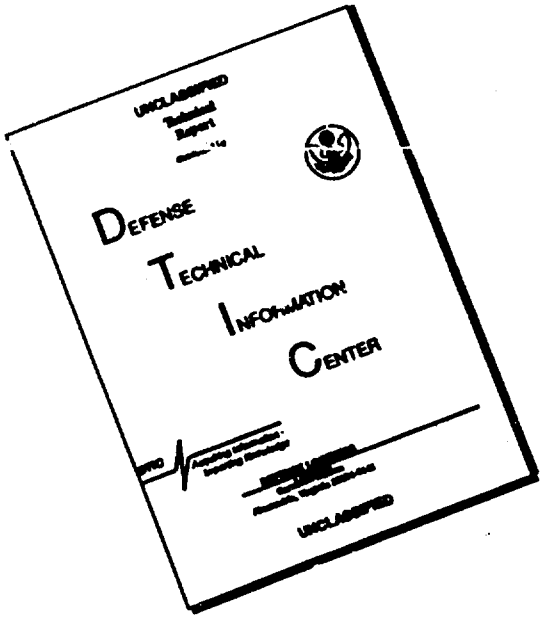

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Werner A. Janssen

Michael J. Surgalla

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORY

Project 1C522301A059

January 1967

FALSE PRECIPITIN BANDS IN OUCHTERLONY GEL DIFFUSION TESTS DUE TO FILTERABLE BACTERIAL FORMS

ABSTRACT

Bands resembling typical antigen-antibody precipitates developed in Ouchterlony gel diffusion tests between sera from certain individual animals and bacteriologically filtered cultures of Aeromonas salmonicida or Vibrio parahemolyticus. After incubation for several days these bands became coarsely granular and progressively more diffuse. Microscopic examination revealed them to be made up of massive numbers of what appeared to be microcolonies of barely visible filamentous bacillary forms. All strains of A. salmonicida and V. parahemolyticus tested produced these filterable forms, which were thermolabile but resistant to a number of other physical and chemical agents. Growth of the filterable forms required a particular concentration of serum and medium, which has so far been achieved only by using the Ouchterlony gel diffusion technique. Reversion of these forms to the typical bacillary form has not been observed. No such forms have been detected in cultures of a variety of other fish and human pathogens.

During a serologic survey of white perch sera for precipitins against various human and fish pathogens we found that about 50% of the serum samples reacted exclusively with Aeromonas salmonicida or Vibrio parahemolyticus. This was quite surprising because precipitins against other Aeromonads or human enteric pathogens were found in only a small percentage of white perch sera.

The implications of this finding were exciting because Aeromonas salmonicida is an important pathogen of trout and salmon, and Vibrio parahemolyticus is a common cause of human food poisoning in Japan because of ingestion of raw fish.

In these studies the agar gel diffusion technique of Ouchterlony was used. [illegible] per cent Oxoid #2 ion agar containing 0.9% NaCl and 0.1 mg of merthiolate per ml was solidified in petri dishes, and appropriate wells were cut in the surfaces. In each test, serum obtained from the heart blood of a white perch was placed in the central wells and various antigens were placed in surrounding wells. The antigens were prepared by growing a particular strain of bacteria for 48 hours at 26 C in Difco brain heart infusion broth containing 1% NaCl. The cultures were sonicated for 10 minutes with a Raytheon sonic oscillator, and then filtered through a 0.20 μ Nalgene filter. One-tenth mg of merthiolate per ml was added and the preparation was stored at 5 C. Each antigen preparation was tested for sterility before use by placing a drop on Difco blood agar base containing 1% NaCl, incubating at 23 C, and observing daily for 2 weeks. The gel diffusion test plates were incubated and observed in the same way.

Figure 1 shows a typical test plate. In this test the white perch serum reacted with both A. salmonicida (well 6) and V. parahemolyticus (well 16). Typical precipitin bands were produced after 24 hours' incubation; however, as seen here these bands began to appear more diffuse than normal after several days' incubation and when observed under 2X magnification on a dissecting microscope the bands had an unusual granular appearance.

Figure 2 is a 7X magnification of the bands between the wells containing the A. salmonicida preparation and serum in the gel plate on Figure 1. At this magnification it can be seen that the bands are made up of a heavy concentration of coarse granules, surrounded by a progressively lighter concentration of granules involving the entire area between the wells. The bands between the V. parahemolyticus preparation and the serum wells were almost identical except that the granules appeared to be smaller. No such granules were observed between any of the other wells. Precipitate forming between antigen and antibody in agar gel appears nongranular and homogenous at this magnification.

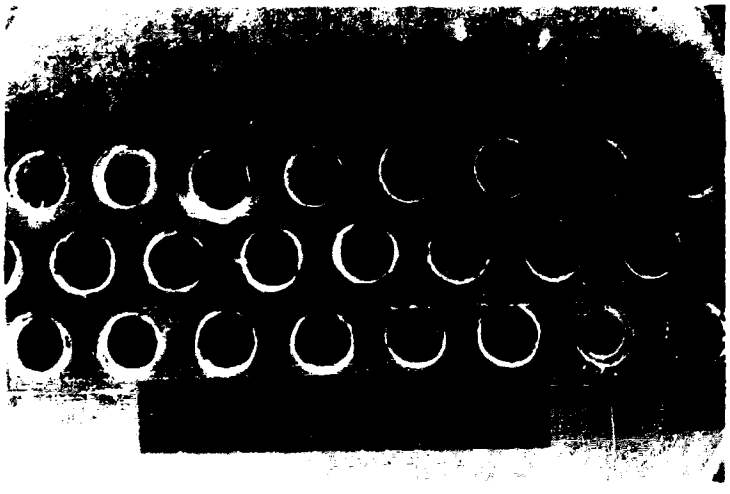

Figure 1. Gel Precipitin Test after 3 Days' Incubation.

Figure 2. Portion of Gel Plate Shown in Figure 1, 7X Magnification.

Figure 3 is a 1200X magnification under phase illumination of the granules making up the bands shown in Figure 2. A small block of agar containing the bands was placed on a microscope slide and flattened as thoroughly as possible under a cover slip. The slide preparation was then sealed with vaspar. The granules are very regular in size and appearance. They are 5 microns in diameter, resemble a ball of cotton, and seem to be made up of a mass of tiny filamentous bacillary forms.

Figure 4 is another photograph of the same preparation. It shows a granule that has been somewhat disrupted to reveal the filamentous nature of the structural elements a little better. As far as we can tell granules on the agar surface look more like this one in that their edges are fuzzier and more irregular than those of subsurface granules.

Figure 5 shows a typical antigen-antibody gel precipitate at 1200X magnification under phase illumination. Note that these precipitate granules are uniformly opaque and very much smaller than the coarse granules in the other figures.

Figure 6 is a slide preparation of the agar gel used in these tests under 1200X magnification and phase illumination. Note the absence of granules or formed elements on the uniformly amorphous background. We have never seen artifacts resembling the tiny fine granules of a true antigen-antibody precipitate or the much larger coarse and structured granules in question on microscopic examination of agar gels in these tests.

Twenty-six strains of A. salmonicida and 17 strains of V. parahemolyticus were tested against a serum sample that had produced the peculiar granular bands described with standard antigen preparations of both of these organisms. Figure 7 shows a typical result after incubation for 1 week at 23 C. All of the strains tested contained the filterable forms. Note the absence of granules between the serum and sterile medium and the denser zone of granules produced by A. salmonicida compared with that of V. parahemolyticus. Bear in mind that these diffuse bands appear only after prolonged incubation at 23 or 37 C, and initially are narrow, well-defined multiple bands during the first 24 to 48 hours of incubation. Even after 1 week sharp bands may still be seen without magnification, but the coarsely granular nature of these bands is easily recognizable under 2X or higher magnification.

So far we have not been able to produce these microcolonies on brain heart infusion agar or Difco PPLO medium containing, in both cases, 1, 10, or 20% positive serum or Difco PPLO serum fraction. They are regularly produced in a diffuse zone around a well containing positive serum in soft agar with added brain heart infusion and a heavy inoculum of filtered A. salmonicida or V. parahemolyticus culture. Sonication of the cultures, filtration, and addition of merthiolate were not necessary for their production. On the other hand, none of these treatments had any effect on their production in the presence of suitable concentrations of proper serum and medium.

Figure 3. Magnification of Granular Zone Shown in Figure 1, 1200X.

Figure 4. Another 1200X Magnification Field of Granular Zone Shown in Figure 1.

Figure 5. Typical Antigen-Antibody Gel Precipitate, 1200X Magnification.

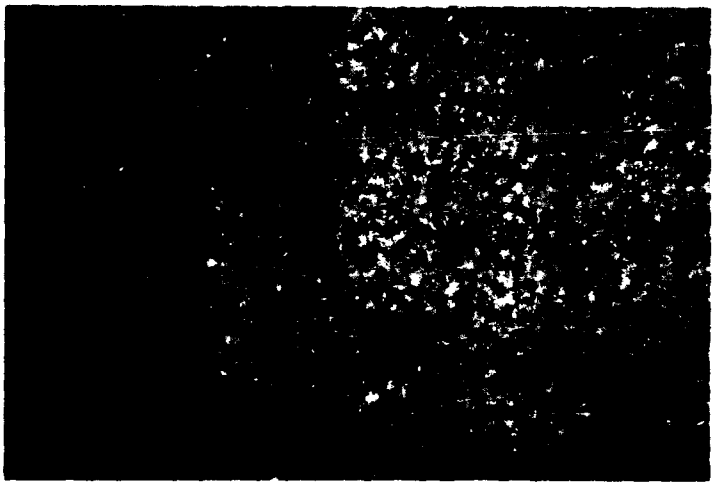

Figure 6. Agar Gel Preparation, 1200X Magnification.

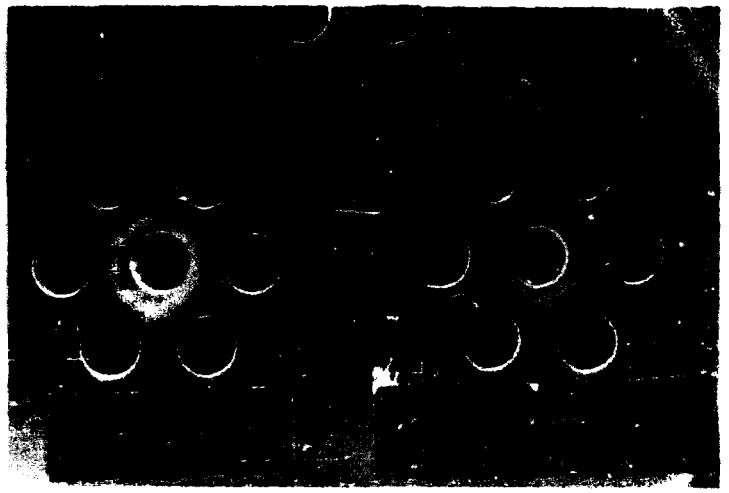

Figure 7. Growth of Filterable Forms on a Gel Precipitin Test Plate after 1 Weeks' Incubation.

Further work has shown that the forms in A. salmonicida and V. parahemolyticus culture responsible for the phenomena described are filterable through any of the bacteriological filters in common use, provided that the filters have never been exposed to acid cleaning solutions. Heating the culture filtrates to 50 C for 1 hour or storage at 5 C for 1 month will destroy the forms, but addition of 0.1% phenol to the antigen preparation or exposure to ultraviolet for 1 hour was without effect, as were streptomycin, penicillin, or sulfadiazine placed in wells near the reaction zone. The forms were acidophilic when stained with Giemsa's stain and readily took up gentian violet.

In addition to certain individual white perch sera, sera from some striped bass, guinea pigs, rats, rabbits, opposssums, and porpoises reacted with either or both of the cultures to produce the coarse granular bands described. The filtrates of A. salmonicida and V. parahemolyticus cultures used in these studies have always proved to be sterile, and typical bacillary forms have not been observed in the media and test systems employed.

In summary, bands resembling typical antigen-antibody precipitates developed in Ouchterlony gel diffusion tests between sera from certain individual animals and bacteriologically filtered cultures of A. salmonicida or V. parahemolyticus. After incubation for several days these bands became coarsely granular and progressively more diffuse. Microscopic examination revealed them to be made up of massive numbers of apparent microcolonies of barely visible filamentous bacillary forms. All strains tested produced these filterable forms, which were thermolabile but resistant to a number of other physical and chemical agents. Growth of the filterable forms required a specific concentration of serum and medium, which has so far been achieved only by the Ouchterlony gel diffusion technique. Reversion of these forms to the typical bacillary form has not been observed. No such forms have been detected in cultures of a variety of other fish and human pathogens.

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*Vibrio parahemolyticus	Food poisoning
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